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Biochemistry

Oxoammonium cation intermediate in the nitroxide-catalyzed dismutation of superoxide

(electron paramagnetic resonance/hydrogen peroxide/superoxide radical/superoxide dismutase/oxoammonium cation)

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ABSTRACT **Dismutation of superoxide has been shown previously to be catalyzed by stable nitroxide compounds. In the present study, the mechanism of superoxide ($\cdot O_2^-$) dismutation by various five-membered ring and six-membered ring nitroxides was studied by electron paramagnetic resonance spectrometry, UV-visible spectrophotometry, cyclic voltammetry, and bulk electrolysis. Electron paramagnetic resonance signals from the carbocyclic nitroxide derivatives (piperidinyl, pyrrolidinyl, and pyrrolinyl) were unchanged when exposed to enzymatically generated $\cdot O_2^-$, whereas, in the presence of $\cdot O_2^-$ and reducing agents such as NADH and NADPH, the nitroxides underwent reduction to their respective hydroxylamines. The reaction of 4-hydroxy-2,2,6,6-tetramethyl-1-hydroxypiperidine (Tempol-H) with $\cdot O_2^-$ was measured and, in agreement with earlier reports on related compounds, the rate was found to be too slow to be consistent with a mechanism of $\cdot O_2^-$ dismutation involving the hydroxylamine as an intermediate. Voltammetric analyses of the carbocyclic nitroxide derivatives revealed a reversible one-electron redox couple at positive potentials. In contrast, oxazolidine derivatives were irreversibly oxidized. At negative potentials, all of the nitroxides studied exhibited a broad, irreversible reductive wave. The rate of $\cdot O_2^-$ dismutation correlated with the reversible midpoint redox potential. Bulk electrolysis at positive potentials was found to generate a metastable oxidized form of the nitroxide. The results indicate that the dismutation of $\cdot O_2^-$ is catalyzed by the oxoammonium/nitroxide redox couple for carbocyclic nitroxide derivatives. In addition to the one-electron mitochondrial reduction pathway, the present results suggest the possibility that cellular bioreduction by a two-electron pathway may occur subsequent to oxidation of stable nitroxides. Furthermore, the cellular destruction of persistent spin adduct nitroxides might also be facilitated by a primary univalent oxidation.**

Stable nitroxide free radicals have found a wide range of applications in biology and medicine. These compounds have been used to monitor intracellular redox reactions (1), oxygen concentration (2), and pH (3), as well as electron paramagnetic resonance (EPR) microscopy of spheroids (4), as contrast agents in magnetic resonance imaging (5), and as probes in EPR imaging (6). Persistent nitroxide adducts resulting from reaction of a precursor nitrone (spin trap) with transient free radical species have been used to detect, characterize, and quantitate the production of free radicals in various *in vitro* and *in vivo* model systems (7). The cellular and *in vivo* pharmacology of stable nitroxides and persistent spin adduct nitroxides has been investigated in detail (8–10). One-electron reduction of stable nitroxides to the corresponding hydroxylamine is the primary metabolic pathway (11–15). Whereas the metabolic fate of spin adduct nitroxides is not clearly understood, oxidative degradation has been suggested (16, 17).

Previous studies have identified a stable five-membered nitroxide, 2-ethyl-2,5,5-trimethyl-3-oxazolidine-1-oxyl (OXANO), as a cell-permeable, nontoxic, catalyst of superoxide ($\cdot O_2^-$) dismutation (18). Subsequent cellular studies established this and other stable nitroxides to be protective agents for cells subjected to oxidative stress induced by agents such as $\cdot O_2^-$, H_2O_2, organic hydroperoxides, and ionizing radiation (19, 20). These initial studies prompted synthesis and screening of various five- and six-membered nitroxides to explore their range as superoxide dismutase (SOD) mimics and the degree of protection they can provide against oxidative damage in mammalian cells (21). The lipophilic piperidinyl nitroxide 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo) ameliorates post-ischemic reperfusion injury (22), and stable nitroxides have been identified as a new class of non-thiol radioprotectors *in vitro* (20). A number of mechanisms for cytoprotection have been advanced; they include oxidation of reduced transition metal ions, detoxification of intracellular radicals such as alkyl, alkoxyl, and alkylperoxyl radicals (19, 20, 23, 24), and direct catalytic removal of $\cdot O_2^-$ by dismutation.

Dismutation of $\cdot O_2^-$ was originally observed by exposing a five-membered cyclic nitroxide to a flux of $\cdot O_2^-$; a lower, yet time-invariant, level of the nitroxide radical EPR signal resulted (18). In the presence of an enzymatic or radiolytic source of $\cdot O_2^-$, either OXANO or its respective hydroxylamine, 2-ethyl-1-hydroxy-2,5,5-trimethyl-3-oxazolidine (OXANOH), gave rise to equivalent levels of residual, steady-state EPR signal. The calculated steady-state ratio ([nitroxide]/[hydroxylamine]) was independent of time, of $\cdot O_2^-$ flux, and of the total concentration of the nitroxide (OXANO· + OXANOH). The proposed mechanism of catalysis entailed initial reduction of nitroxide by $\cdot O_2^-$ to form the corresponding hydroxylamine and subsequent reoxidation of the hydroxylamine by $\cdot O_2^-$. However, when piperidinyl nitroxide derivatives were allowed to react with $\cdot O_2^-$, no decrease in EPR signal levels was detected (25). To account

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Abbreviations: EPR, electron paramagnetic resonance; Tempo, 2,2,6,6-tetramethylpiperidine-1-oxyl; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; Tempol-H, 4-hydroxy-2,2,6,6-tetramethyl-1-hydroxypiperidine; Tempamine, 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl; Tempone, 4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl; OXANO, 2-ethyl-2,5,5-trimethyl-3-oxazolidine-1-oxyl; OXANOH, 2-ethyl-1-hydroxy-2,5,5-trimethyl-3-oxazolidine; CHDO, 2-spirocyclohexane-5,5-dimethyl-3-oxazolidine-1-oxyl; DTPA, diethylenetriaminopentaacetic acid; SOD, superoxide dismutase; CAT, catalase; CV, cyclic voltammetry; $\cdot O_2^-$, superoxide; HX, hypoxanthine; XO, xanthine oxidase; NHE, normal hydrogen electrode.

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for the persistence of the EPR signal, rapid reoxidation of the hydroxylamine was postulated (25).

In the present study, the mechanism of $\cdot O_2^-$ reactions with nitroxides and hydroxylamines has been investigated using EPR spectrometry, cyclic voltammetry (CV), and bulk electrolysis. Rapid reoxidation of the hydroxylamine form of Tempol by $\cdot O_2^-$ was not observed, and, in contrast to the previously proposed mechanism, evidence is presented herein that demonstrates that dismutation of $\cdot O_2^-$ can proceed by a mechanism involving an intermediate oxoammonium cation.

MATERIALS AND METHODS

Chemicals. Xanthine oxidase (XO), nicotinamide adenine dinucleotide reduced (NADH) and oxidized (NAD^+), and catalase (CAT) were purchased from Boehringer Mannheim. Diethylenetriaminopentaacetic acid (DTPA), 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl, 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl, 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl, 3-cyano-2,2,5,5-tetramethylpyrrolidine-1-oxyl, 3-aminomethyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl, Tempo, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol), and 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempamine) were purchased from Aldrich. 4-Oxo-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempone) was from Molecular Probes. The hydroxylamine form of Tempol [4-hydroxy-2,2,6,6-tetramethyl-1-hydroxypiperidine (Tempol-H)] and OXANO were prepared as described earlier (18, 20). EPR analyses were conducted as described (18).

Electrochemical Analysis. Redox properties of nitroxides were investigated at 23°C by CV and square wave voltammetry techniques using a BAS 100A electrochemical analyzer (Bioanalytical Systems, West Lafayette, IN). Aqueous solutions of the nitroxides were analyzed using a vitreous carbon working electrode and a platinum auxiliary electrode. The reference was a Ag/AgCl electrode, calibrated against a primary saturated calomel electrode (SCE). Data from CV were collected over several sweep cycles, each from −1000 mV to +1000 mV and back to −1000 mV versus the reference Ag/AgCl. Square wave voltammetry was performed according to the method of Osteryoung and Osteryoung (26) at a frequency of 15 Hz and amplitude of 25 mV stepped at 4-mV increments, with potential sweeps in negative and positive directions between +1000 mV and −1000 mV versus the reference electrode. All potentials are reported versus the normal hydrogen electrode (NHE) using a value of +0.2415 V as the standard reduction potential of SCE versus NHE. In bulk electrolysis experiments, an electrochemical reactor similar to that described by Miner and Kissinger (27) was used. The cell consisted of a working electrode of graphite packed inside a porous Vycor glass tube (5-mm i.d.), through which the test solutions were pumped (≈2 ml/min). An outer glass cylinder, with separate electrolyte, contained the platinum auxiliary electrode and a Ag/AgCl reference electrode. Controlled potential electrolysis was performed with a CV-27 Potentiostat (Bioanalytical Systems). The reactor effluent was analyzed directly by EPR.

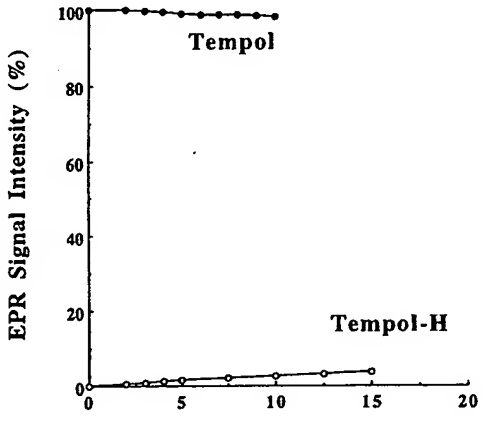

FIG. 1. EPR signal intensity of Tempol followed as a function of time of exposure of Tempol, 100 μM (closed symbols), and Tempol-H (open symbols) to $\cdot O_2^-$ generated by HX (2.5 mM)/XO (0.02 unit/ml) in air-saturated phosphate buffer (50 mM, pH 7.8) in the presence of CAT (100 units/ml) and DTPA (50 μM). The Tempol-H oxidation to Tempol was completely inhibited by SOD (100 units/ml).

RESULTS

EPR Analysis of Nitroxide Reaction with $\cdot O_2^-$. The reaction of $\cdot O_2^-$ with the nitroxide Tempol and its hydroxylamine form (Tempol-H) was studied by exposure to $\cdot O_2^-$ generated by the aerobic reaction of hypoxanthine (HX) and XO. The EPR signal of Tempol was monitored as a function of reaction time, and the results are shown in Fig. 1. No significant decrease in the EPR signal was observed. Higher fluxes of $\cdot O_2^-$, generated in reactions with higher XO levels, also failed to decrease the EPR signal. Similar results were found with other pyrrolidinyl, pyrrolinyl, and piperidinyl nitroxides. These findings stand in contrast with the observed decrease

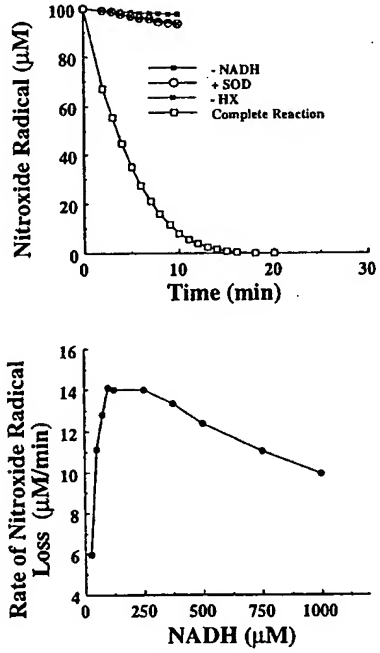

FIG. 2. (*Upper*) Time dependence of EPR signal (open squares) of Tempol following the addition of 0.02 unit of XO per ml to 50 mM phosphate buffer (pH 7.8) containing 50 μM DTPA, 2.5 mM HX, 100 units of CAT per ml, and 100 μM Tempol in air at room temperature in the presence of 250 μM NADH. Control experiments are as follows: complete reaction plus 300 units of SOD per ml (open circles); without hypoxanthine (×); without NADH (closed squares). (*Lower*) Initial rates of loss of Tempol EPR signal as a function of [NADH] following the addition of 0.02 unit of XO per ml to 50 mM phosphate buffer (pH 7.8) containing 50 μM DTPA, 2.5 mM HX, 100 units of CAT per ml, and 100 μM Tempol in air at room temperature.

of the EPR signal when oxazolidine nitroxide derivatives are allowed to react with $\cdot O_2^-$ (18, 25).

To test for oxidation of the hydroxylamine by $\cdot O_2^-$, Tempol-H was allowed to react under similar conditions. As shown in Fig. 1, the nitroxide radical formation was exceedingly slow. The slow rate of reaction of Tempol-H with $\cdot O_2^-$ was independently determined by the ferricytochrome *c* reduction assay and found to be $4 \times 10^2\ M^{-1}\cdot s^{-1}$. This value is two orders of magnitude below that estimated for the reaction of $\cdot O_2^-$ with Tempol (25) and in the same order of magnitude estimated for Tempo-H (28). Thus the previously proposed mechanism of $\cdot O_2^-$ dismutation involving an intermediate hydroxylamine is not consistent with these observed rates.

Although a clear demonstration of the hydroxylamine formation from the piperidinyl nitroxides has been shown in $\cdot O_2^-$-dependent reactions, sulfhydryl compounds were required as reducing agents (29). Since thiols can function as either one- or two-electron donors, we have chosen to test the reaction using a reducing agent such as NADH, which is an obligate two-electron donor. Shown in Fig. 2 are the results of the reaction of Tempol with $\cdot O_2^-$ (generated by the HX/XO reaction) in the presence of NADH. A time-dependent decrease in the EPR signal intensity was observed. After completion of the reaction, addition of the one-electron oxidant ferricyanide restored the EPR signal to its original level. All other nitroxides tested also underwent reduction in the presence of NADH and $\cdot O_2^-$. Control reactions in which HX was omitted or to which SOD was added showed negligible loss of EPR signal (Fig. 2 *Upper*). Likewise, no direct reaction of nitroxides with NADH was detected. The rate of nitroxide reduction was dependent on NADH concentration, as shown in Fig. 2 *Lower*. The reduction was supported by either NADH or NADPH but not by NAD^+ or $NADP^+$. Concomitant oxidation of NADH to NAD^+ was verified spectrophotometrically. Addition of SOD inhibited the loss of nitroxide EPR signal (as shown in Fig. 2 *Upper*) and NADH oxidation. These results suggest a two-electron reduction of an oxidized form of the nitroxide produced initially during $\cdot O_2^-$ dismutation.

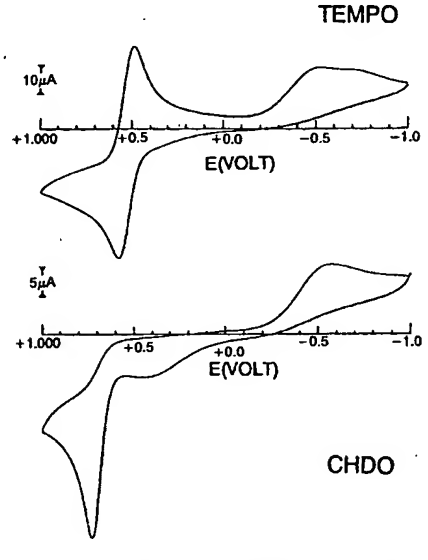

FIG. 3. CV of nitroxides in aqueous solution. CV was carried out on anaerobic 2.0 mM nitroxide solutions in phosphate buffer (pH 7.4) containing 0.154 M NaCl, at 0.200 V/s (see text). CHDO, 2-spirocyclohexane-5,5-dimethyl-3-oxazolidine-1-oxyl.

CV. For catalytic dismutation of $\cdot O_2^-$ by a nitroxide/oxoammonium cation couple, a redox potential in the range of −0.33 V ($O_2/\cdot O_2^-$) to +0.89 V ($\cdot O_2^-/H_2O_2$) would be required (30). Therefore, the redox properties of various five- and six-membered nitroxides were investigated by CV and square wave voltammetry. Voltammogramic data from analysis of Tempo are presented in Fig. 3 *Upper*. The results show a reversible one-electron redox reaction in the region of positive potential attributed to the nitroxide/oxoammonium couple (31). In the region of negative potential, an irreversible reduction wave was detected, which possibly represents the generation of the hydroxylamine. The pattern shown in Fig. 3 *Upper* was typical of results from the six-membered and five-membered carbocyclic nitroxide derivatives tested. In contrast, the five-membered ring heterocyclic oxazolidinyl derivatives underwent irreversible oxidation, as shown in Fig. 3 *Lower*. Redox midpoint potentials measured for the various nitroxide derivatives are listed in Table 1. As shown for Tempo, Tempol, and Tempamine, which are known to catalyze $\cdot O_2^-$ dismutation, the redox potentials of the nitroxide/oxoammonium couple are in the range +0.722 V to 0.872 V. Furthermore, the rates of EPR signal loss in the presence of $\cdot O_2^-$ and NADH generally correlated with the midpoint potentials, as shown in Table 1. This type of correlation

Table 1. Oxidation/reduction midpoint potentials of various nitroxide radicals in aqueous solution

Compound	Apparent midpoint potential,* mV	Method(s)†	Reduction rate‡
Tempo	722	CV, OSWV	1.00
Tempol	810 (720)	CV, OSWV	0.88
Tempamine	826 (872)	CV	—
Tempone§	913 (912)	CV, OSWV	0.33
3-Carboxyproxyl	792 (772)	CV, OSWV	0.45
3-Aminomethyl-proxyl	853	CV	—
3-Carbamoylproxyl	861 (872)	CV, OSWV	0.17
3-Cyanoproxyl	976 (982)	CV, OSWV	0.02
3-Carbamoyl-3-pyrroline	966 (972)	CV	0.03
OXANO¶	960‖	OSWV	—
CHDO¶	900‖	CV, OSWV	—
Ferri/ferrocyanide	410, 408**, 430††	CV	

Potentials are reported versus NHE.

*Values in parentheses were obtained from ref. 32 and were adjusted by +242 mV for comparison based on NHE.

†OSWV, Osteryoung square wave voltammetry. Where indicated, both methods were used, and in all cases results from both methods agreed to within 4 mV for compounds where oxidation was freely reversible.

‡Reduction rates of nitroxides (100 μM) by $\cdot O_2^-$ generated by XO (0.02 unit/ml) in air-saturated phosphate buffer (50 mM, pH 7.8) containing 50 μM DTPA and CAT (100 units/ml) in the presence of 250 μM NADH. The rates are normalized with respect to the rate for Tempo.

§Irreversible oxidation at low sweep rates.

¶Irreversible oxidation under all conditions tested up to 50 V/s.

‖Pseudo midpoint estimation from oxidation wave peaks (after subtraction of 30 mV) in CV carried out at scan rates of 2–20 V/s. Peak potentials of scans at 0.020 V/s were roughly 80 mV less than values at 20 V/s. For CHDO square wave voltammetry peaks were found at +895 mV in scans done from positive to negative direction. The peak currents were much weaker than in scans done in the oxidative direction (which show a +875 mV peak). However, it is reasonable in cases where rapid chemical reaction of the oxidized species removes it from the electrode reaction that the pseudo midpoint is provided by the peak potential from the negative scan, rather than by scans in the oxidative reaction.

**Data from ref. 33.

††Data from ref. 34.

would be expected if an initial one-electron oxidation was required prior to reductive loss of the EPR signal.

Bulk Electrolysis. To extend the results obtained from CV, controlled-potential bulk electrolysis was carried out on the nitroxides Tempo and Tempol. Electrochemical oxidation of 0.2 mM aqueous solutions of these nitroxides at 0.810 V (NHE) resulted in extensive (88–94%) loss of the EPR signal. In the case of Tempol, the residual EPR signal resulted in part from formation of a second nitroxide, with a nitrogen hyperfine coupling of a_N = 16.0 G (1 G = 0.1 mT), tentatively identified as Tempone. In contrast, when the unsubstituted Tempo was electrolyzed, there was no evidence for the formation of a second nitroxide. When the oxidized solutions were immediately (<5 min) subjected to reduction at +0.64 V, recovery of the nitroxide signal was substantial (>75% in the case of Tempo). Therefore, an EPR silent, metastable oxidized form of the nitroxide was generated at potentials predicted from the CV experiments.

DISCUSSION

The widespread use of stable nitroxides and persistent spin adduct nitroxides in biology suggests a need to understand better the factors influencing the stability of these compounds in the cellular milieu. The present study strongly suggests that stable nitroxides (piperidinyl and pyrrolidine derivatives) catalyze the dismutation of $\cdot O_2^-$ by utilizing the oxoammonium cation/nitroxide redox couple. The $\cdot O_2^-/H_2O_2$ couple has a redox potential of +0.89 V, and the catalytic dismutation rate was found to be directly related to the midpoint redox potential of the corresponding nitroxide. Tempo (0.722 V), Tempol (0.810 V), and Tempamine (0.826 V) have catalytic rates of $\cdot O_2^-$ dismutation of 1.2×10^5 $M^{-1}\cdot s^{-1}$, 6.5×10^4 $M^{-1}\cdot s^{-1}$, and 6.5×10^4 $M^{-1}\cdot s^{-1}$, respectively (25). The doxyl (oxazolidine-1-oxyl) derivatives dismutate $\cdot O_2^-$ by utilizing the hydroxylamine/nitroxide pair (18) and consequently catalyze the reaction at 1/100th the rate of the piperidinyl and proxyl (pyrrolidine-1-oxyl) nitroxides. All nitroxides studied with the exception of the doxyl derivatives exhibited reversible electrochemical behavior when oxidized. The doxyl derivatives, OXANO and CHDO, were not reversible in either direction (Fig. 3 *Lower*). The reactions of nitroxides with $\cdot O_2^-$ reveal that the piperidinyl and the proxyl derivatives are reduced in an $\cdot O_2^-$-dependent manner only in the presence of two-electron donors such as NADH and NADPH. The reduction products of these nitroxides are the corresponding hydroxylamines. These results suggest that the initial reaction with $\cdot O_2^-$ is a one-electron oxidation to the oxoammonium cation. This transient species either may be reduced back to the nitroxide by $\cdot O_2^-$ or may react with NADH or NADPH to form the corresponding hydroxylamine. Additional support for the proposed oxoammonium intermediate was obtained by bulk electrolysis experiments, wherein the nitroxide EPR signal was found to decrease at potentials predicted from CV.

During dismutation of $\cdot O_2^-$, high steady-state levels of piperidinyl and proxyl derivative nitroxides remain. This suggests that the reduction of the oxoammonium cation by $\cdot O_2^-$ is relatively fast. The doxyl derivatives, on the other hand, are directly reduced by $\cdot O_2^-$ to their hydroxylamines (18, 25). The irreversible electrochemical oxidation of the doxyl derivatives suggests a mechanism for dismutation of $\cdot O_2^-$ that would not involve catalysis by an oxidized nitroxide intermediate. Based on the observations, the following reactions are proposed for the nitroxide-mediated dismutation of $\cdot O_2^-$.

$$RR'NO\cdot + \cdot O_2^- + 2H^+ \xrightarrow{k_1} [RR'NO^+] + H_2O_2. \quad [1]$$

$$[RR'NO^+] + \cdot O_2^- \xrightarrow{k_2} RR'NO\cdot + O_2. \quad [2]$$

Reaction 1 involves the oxidation of nitroxide (RR'NO·) to form the oxoammonium cation intermediate [RR'NO$^+$]. This intermediate is subsequently reduced by $\cdot O_2^-$ (reaction 2) to reform nitroxide, resulting in the overall dismutation of $\cdot O_2^-$, as shown in reaction 3.

$$2 \cdot O_2^- + 2H^+ \xrightarrow{k_{cat}} O_2 + H_2O_2. \quad [3]$$

Although direct evidence for reaction of the oxoammonium cation with $\cdot O_2^-$ (reaction 2) was not obtained, in biological systems this intermediate may be expected to react with many endogenous one- and two-electron reducing agents. Thus the pathway for $\cdot O_2^-$ dismutation *in vivo* would not necessarily involve direct reduction of $\cdot O_2^-$ (reaction 2) as the major route for regeneration of the cyclic nitroxide. In view of the instability of the oxoammonium cation (ref. 35; also noted in our bulk electrolysis experiments) it would appear that regeneration of the nitroxide would occur predominantly by reaction of the oxoammonium with endogenous substrates other than $\cdot O_2^-$. This raises the possibility of deleterious reactions with critical biomolecules if repair (rereduction) mechanisms are too slow to prevent subsequent irreversible processes.

In the absence of two-electron reductants, reactions 1 and 2 do not contribute to hydroxylamine formation. In the presence of two-electron donors such as NADH (under conditions of [NADH] >> [$\cdot O_2^-$]), competition for the oxoammonium cation may occur and decrease the product from reaction 2. The competitive reaction would involve direct two-electron reduction of the oxoammonium cation as follows:

$$NADH + [RR'NO^+] \rightarrow NAD^+ + RR'NOH. \quad [4]$$

Since complete reduction of the nitroxide was observed with excess [NADH], any reoxidation of the hydroxylamine would be slow, consistent with the results shown in Fig. 1.

Metabolic processes result in the reduction of nitroxides to their hydroxylamines. The efficiency of reduction was found to be faster in hypoxic cells (11). More detailed analyses of the cellular components responsible for reduction showed that the cytosolic fractions of cells reduce nitroxides by utilizing ascorbic acid (15), whereas the microsomal fractions mediate the reaction through enzymatic processes (14). It has been suggested that the primary organelle responsible for intracellular reduction of nitroxides is the mitochondrion (14). At the cellular level it has been found that reduction is faster for six-membered piperidinyl nitroxides than for five-membered nitroxides (32). The hydroxylamines, on the other hand, are oxidized by cellular processes only under aerobic conditions at the level of cytochrome *c* oxidase (11). In a recent report (32), midpoint potentials for the one-electron oxidation of various nitroxide derivatives have been correlated to the substituent constants for inductive effects on the ring. It was suggested that midpoint reduction potential, in addition to the lipophilicity, might influence the intracellular stability of nitroxides. Fig. 4 demonstrates that, subsequent to oxidation, the two-electron reduction of the six-membered nitroxides (Tempo, Tempol, and Tempone) correlates with the midpoint oxidation potential, whereas, when Table 1 is examined, the influence of ring size on reduction rate can be seen when different ring sizes with similar midpoint oxidation potentials are compared. The trends shown in Table 1 have also been observed for microsomal and mitochondrial reduction of nitroxides (10, 32, 36). Increases in endogenous cellular reducing equivalents would be expected to facilitate the reaction product through reaction 4. When considering

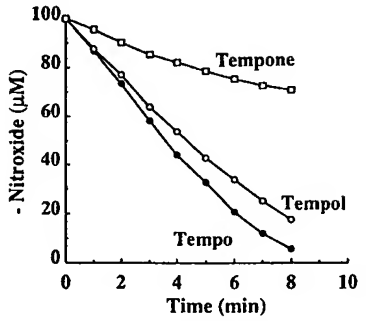

FIG. 4. Plot of the $\cdot O_2^-$-induced reduction rates of various piperidine nitroxides (100 mM) in the presence of NADH (250 μM). A reaction containing HX (2.5 mM) and XO (0.02 unit/ml) in air-saturated phosphate buffer (50 mM, pH 7.8) containing 50 μM DTPA and CAT (100 units/ml) was used to generate $\cdot O_2^-$. The rates of reduction were measured following the addition of XO by continually monitoring the EPR signal intensities of the nitroxides. Closed circles, Tempo; open circles, Tempol; and open squares, Tempone.

the special case of cellular hypoxia, reaction 4 may become predominant because molecular oxygen is no longer the primary electron sink and, therefore, the oxidized nitroxide no longer competes with molecular oxygen for reducing equivalents (32). For the case of persistent spin adducts in cellular environments, the metabolism is apparently more complex (9). It is known that nitroxides, formed from the addition of unstable radicals to a variety of spin traps, are destroyed by recombination with other radical species (17). In cells, the spin adduct nitroxides have short lifetimes and are rapidly metabolized to species other than the hydroxylamines (16).

The present study demonstrates a pathway for $\cdot O_2^-$ dismutation that is distinct from the pathway involving one-electron reduction of nitroxides. It is proposed that during reaction of $\cdot O_2^-$ with piperidinyl and proxyl nitroxides in the presence of reducing agents the generation of the hydroxylamine results from a two-electron reduction of the corresponding oxoammonium cation intermediate. Moreover, the results suggest the possibility that cellular destruction of persistent spin adduct nitroxides may be facilitated by primary univalent oxidation. Factors that influence either the redox state of cells or concentrations of oxygen and $\cdot O_2^-$ may well determine which nitroxides are chosen as probes of cellular metabolism or as NMR contrast agents. Ultimately, such factors should also aid in the choice of the appropriate nitroxide to be used for *in vivo* EPR imaging.

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